Separation of Lymphocytes and Macrophages from Suspensions of Guinea Pig Peritonitis Exudate Cells Using Programmed Gradient Sedimentation

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A VARIETY OF TISSUES AND CELL TYPES have been used to study the different aspects of delayed hypersensitivity *in vitro*.[1] In 1962, George and Vaughan[2] introduced the macrophage inhibition test, an assay for delayed hypersensitivity *in vitro* involving the inhibition of peritonitis cell migration from capillary tubes. David *et al*[3] utilized this technic to show that as few as 2.5% peritoneal exudate cells from a sensitized guinea pig inhibit the migration of a mixture of peritoneal cells from sensitized and "normal" animals. The macrophage inhibition test has been used to demonstrate sensitization to brain extracts in animals with experimental autoimmune (allergic) encephalomyelitis,[4] in the detection of tumor-specific antigens,[5] to prove immunity resulting from virus infection[6] and in a host of other experimental situations.[7-9]

Many variables in this *in vitro* model have not been critically evaluated, particularly the type of mineral oil used in eliciting the exudative peritoneal response. Mineral oils differ in their biologic effects. In studies with Balb/c mice, Potter and his colleagues[10] have shown that a variety of mineral oils differ markedly in their ability to induce myelomas when given intraperitoneally. In using the white mineral oil Drakeol 6VR in the preparation of vaccine emulsions, Wilner *et al*[11] found that a single mineral oil varies considerably from lot to lot with respect to both toxicity and adjuvant potentiation. Such variability is understandable when it is considered that crude mineral oils consist of literally hundreds of different paraffins and naphthenes.[12] These inconsistencies in composition and biologic activity led us to seek substitutes for the crude mineral oils that would eliminate the

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difficulty of chemical definition. Chemically pure hydrocarbon compounds found in mineral oil were selected that represent a range of toxicity and physical characteristics. Having standardized our experimental conditions by specifying the oil used to elicit the peritonitis and the age, sex, diet and housing of the guinea pig (each of these conditions proved to be a significant variable in preliminary studies), we could proceed to select suitable oils for the induction of guinea pig peritonitis and to develop methods for the purification of lymphocytes and macrophages from guinea pig peritonitis cells. Zbar *et al*[13] recently reported the purification of lymphocytes and macrophages from oil-induced guinea pig peritonitis exudate by using density gradient sedimentation; they obtained macrophages in 87% purity and lymphocytes in 45% purity after separation on discontinuous albumen gradients. We now report a method for obtaining macrophages in 98.5–100% purity and lymphocytes in 99–100% purity using a computer integration of the differential sedimentation equation to predict the gradient design and the speed and duration of centrifugation that will permit purification of the desired cell types. The macrophages and lymphocytes in the peritoneal exudates that are elicited by the different oils also are compared with respect to differences in density.

Materials and Methods

Previous reports have included detailed descriptions of the theory of gradient sedimentation as applied to mammalian cells,[14–16] the experimental procedures used in this work [17] and the results of numerous experiments involving the separation of specific cells, including the separation of rabbit thymocytes from Ehrlich ascites tumor cells,[14] osteoclasts from rat metaphyseal cell suspensions,[18] human leukemia cells from peripheral blood,[19] malignant cells from stromal cells in disaggregated solid tumors [20] and mast cells from each other according to degree of differentiation.[21] Therefore, a detailed discussion of the methods and theory will be omitted from this report.

Hydrocarbon

N-hexadecane and 2,6,10,14-tetramethylpentadecane were purchased from the Aldrich Chemical Co, Inc. Squalane (2,6,10,15,19,23-hexamethyltetracosane) was purchased from Eastman Organic Chemicals. Drakeol 6VR was obtained from the Pennsylvania Refining Co; Squibb Mineral Oil, from ER Squibb & Sons Inc. Marcol 52, previously marketed as Bayol F, was donated by the Humble Oil & Refining Co.

Guinea Pigs

Male, Hartley strain, white guinea pigs each weighing 200 g were purchased from Davidson's Mill Farm in North Brunswick, New Jersey. The animals were housed, 2 per cage, in 19 × 10½ × 5 inch polypropylene cages containing an approximately 1-inch layer of wood shavings (Sani-Chips, WF Fisher). They were given Purina Guinea Pig Chow and fresh lettuce daily with water and hay *ad libitum*. Animals were weighed twice weekly for a minimum of 3 weeks before being

used in experiments to assure that they were gaining weight normally. At the time of the experiment, the animals were transferred individually to stainless steel, wire-bottomed, 18 × 12 × 12 inch cages. The feeding procedure was continued as described above.

Hydrocarbon Injection

Hydrocarbons were injected when the guinea pig reached a weight of between 360 and 440 g (8 weeks). Thirty milliliters of pure hydrocarbon or mineral oil was sterilized by autoclaving for 20 minutes at 125 C. The guinea pig was anesthetized with ether and given 25 ml of hydrocarbon intraperitoneally.

Collection and Counting of Cells

Four days (96 ± 3 hours) after receiving the hydrocarbon, the guinea pig was etherized and exsanguinated by decapitation. The peritoneal exudate cells were collected by washing the peritoneal cavity once with 40 ml and twice with 20 ml of Joklik tissue culture medium (Grand Island Biological Co) containing 10% fetal calf serum and heparin (2 units/ml). All solutions were kept at ice-bath temperatures. The combined peritoneal washings were placed in a separatory funnel for 5 minutes, after which the aqueous phase was withdrawn from the bottom of the funnel. Cell counts were done using hemocytometer chambers. Slides were prepared for microscopic examination using the Cytocentrifuge (Shandon Scientific Co).

Differential Counting of Harvested Cells

Differential counts were done, counting at least 500 cells from each experimental animal. Cells were classified as macrophages, lymphocytes, neutrophils, red blood cells and lysed cells.

Density Gradients

Density gradients of sterile Ficoll (polysucrose, average mol wt 400,000; Pharmacia Fine Chemicals) in Joklik tissue culture medium (Grand Island Biological Company) were prepared using the technic and two-chambered gradient maker that were described and pictured previously.[17] Density gradients for both isopycnic and rate-zonal sedimentation experiments were constructed in such a way that the densities of the gradients varied as linear functions of the distance from the center of revolution. The density gradients for isopycnic cell separations varied linearly from 5.5% w/w Ficoll at the sample-gradient interface, 18.2 cm from the center of revolution, to 43.0% w/w Ficoll at the gradient-cushion interface, 26.0 cm from the center of revolution. The density gradients for rate-zonal separations varied linearly from 2.6% w/w Ficoll at the sample-gradient interface, 13.7 cm from the center of revolution, to 5.5% w/w Ficoll at the gradient-cushion interface, 26.0 cm from the center of revolution. A 10-ml cushion of 43.5% w/w Ficoll was placed immediately beneath the density gradients for both isopycnic and rate-zonal separations; both kinds of gradients were contained in 100-ml polycarbonate centrifuge tubes (No. 2806, International Equipment Company).

Peritonitis Cells for Starting Sample Suspensions

In both isopycnic and rate-zonal cell separation experiments, a 7-ml aliquot of the 80-ml washings from a single guinea pig was used as the starting sample suspension which was layered over each gradient. The starting sample suspension for each gradient contained 11–24 × 10^6 cells, depending on the concentration of peritonitis

cells recovered from the peritoneal exudate. The separations achieved in both isopycnic and rate-zonal cell separation experiments were independent of the number of cells in the starting sample suspensions over the examined range of cell concentrations.

In some two-step experiments with peritonitis cells resulting from the injection of squalane, both rate-zonal and isopycnic separations were done. In these experiments, the fraction that contained the lymphocyte peak from the rate-zonal gradient was layered over an isopycnic gradient; and isopycnic centrifugation was then carried out at 800 *g* (measured at the sample-gradient interface, 18.2 cm from the center of revolution) for 90 minutes at 4.0 C.

Centrifugation

In isopycnic centrifugation experiments (Table 1), centrifugation was carried out at 800 *g* (measured at the sample-gradient interface, 18.2 cm from the center of revolution) for 90 minutes at 4.0 C. Preliminary rate-zonal sedimentation experiments were carried out on the described rate-zonal gradients for short periods of time at 18.7 *g*. The modal populations of lymphocytes, macrophages and neutrophils were located. These locations, together with the modal densities as determined from isopycnic sedimentation, were substituted in the computer integration of the differential sedimentation equation and used to calculate effective diameters for each

Table 1. Results of One-Step, Isopycnic Separations of Cells Induced by Different Oils

Before and after separation	Centrifugation		Type of cell		
	Force (g)	Duration (min)	Macrophages	Lymphocytes	Neutrophils
			Tetramethylpentadecane		
Sample before separation (%)*			47.8–63.2	12.0–24.9	16.7–35.3
Treatment	800	90			
Purity after separation (%)			88.5–93.0	12.5–19.0	83.0–99.0
Purest fraction No.			2–5	7–8	13
			Squalane		
Sample before separation (%)*			57.5–84.1	8.2–19.4	6.5–23.9
Treatment	800	90			
Purity after separation (%)			98.5–99.5	42.0–45.0	69.0–87.5
Purest fraction No.			1–2	8±1	11–12
			Squibb Mineral Oil		
Sample before separation (%)*			68.9–90.7	6.5–12.5	15.0–20.3
Treatment	800	90			
Purity after separation (%)			100.0–100.0	36.0–63.5	52.5–68.5
Purest fraction No.			1–5	8–12	13

* 95% confidence interval.

of the modal populations as described previously.[6] The effective diameters and densities of the modal populations were then substituted in the computer integration of the differential sedimentation equation, and rate-zonal separation experiments were simulated in order to determine (1) if the employed rate-zonal gradient would permit separation of the various cell types and (2) the optimal speed and duration of centrifugation for cell purification. Rate-zonal experiments that were simulated on several alternative gradients failed to improve the results obtained from simulated experiments on the gradient that was ultimately used in the laboratory experiments reported in this paper. For the rate-zonal gradient described above, optimal durations and forces of centrifugation for the one-step purifications of lymphocytes and macrophages from the various peritonitides are listed together with the resulting purifications in Table 2.

Collection and Counting of Fractions

After centrifugation, fractions were collected, using the gradient tapping cap described and pictured previously.[17] Fraction 1 was comprised of the 7-ml starting

Table 2. Results of One-Step, Rate-Zonal Separations of Cells Induced by Different Oils

Before and after separation	Centrifugation		Type of cell		
	Force (g)	Duration (min)	Macrophages	Lymphocytes	Neutrophils†
Tetramethylpentadecane					
Sample before separation (%)*			47.8–63.2	12.0–24.9	16.7–35.3
Treatment	121	12			
Purity after separation (%)			73.5–79.0	68.0–77.0	45.5–97.0
Purest fraction No.			14 ± 1	10 ± 1	21†
Squalane					
Sample before separation (%)*			57.5–84.1	8.2–19.4	6.5–23.9
Treatment	97.3	16			
Purity after separation			88.5–95.0	55.0–84.5	9.0–62.0
Purest fraction No.			2–2	10	21†
Squibb Mineral Oil‡					
Sample before separation (%)*			68.9–90.7	6.5–12.5	15.0–20.3
Treatment	54.7	30			
Purity after separation (%)			94.0–96.5	78.5–83.0	21.0–32.5
Purest fraction No.			1–2	10	21†

* 95% confidence interval.

† A large proportion of neutrophils were involved in cell aggregates; since aggregates have effective diameters, they sediment rapidly to the gradient-cushion interface in fraction No. 21.

‡ The results reported here were obtained with batch No. 9B060 and are not valid for some other batches of Squibb mineral oil.

sample volume. Fraction 2, containing the sample-gradient interface, and successive fractions from the gradient and cushion were 4-ml fractions. Cell counts were performed on all fractions, using hemocytometer chambers. Slides for microscopic examination were prepared using the Cytocentrifuge (Shandon Scientific Company) and stained with the Wright-Giemsa technic. Differential counts were done, counting 200 cells from each gradient fraction.

Results

Sample Composition

An analysis of the mean cellular compositions and 95% confidence intervals that are characteristic of the peritonitides elicited by each of the respective oils under the described conditions is shown in Table 3.

Table 3. Cellular Composition of Peritonitis*

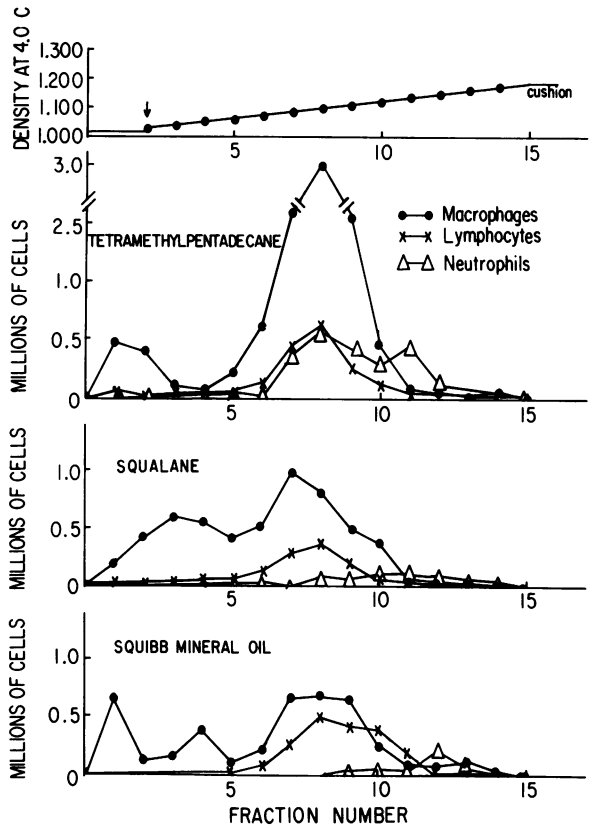
Name	Type of cell (%)		
	Macrophages	Lymphocytes	Neutrophils
Chemicals			
Hexadecane			
Mean	22.3	7.4	70.3
SD	7.9	2.1	9.7
95% CI	14.1–30.1	4.8–10.0	68.5–82.1
Tetramethylpentadecane			
Mean	55.5	18.5	26.0
SD	6.3	5.2	7.6
95% CI	47.8–63.2	12.0–24.9	16.7–35.3
Squalane			
Mean	70.8	13.8	15.4
SD	10.9	4.5	7.1
95% CI	57.5–84.1	8.2–19.4	6.5–23.9
Mineral Oils			
Drakeol 6VR			
Mean	64.2	7.6	28.2
SD	8.7	2.1	10.2
95% CI	53.6–74.9	4.9–10.3	15.6–40.5
Squibb			
Mean	79.8	9.3	10.9
SD	8.9	2.3	7.7
95% CI	68.9–90.7	6.5–12.5	15.0–20.3
Marcol 52			
Mean	61.7	11.0	27.3
SD	4.9	3.8	8.6
95% CI	55.0–67.7	6.4–15.6	16.6–37.5

* The means, standard deviations (SD), and 95% confidence intervals (95% CI) were calculated using the data obtained from 5 animals for each mineral oil and pure hydrocarbon ($n = 5$).

The isopycnic separations reported in Table 1 and the two rate-zonal separations using Squibb mineral oil were performed using the same batch of Squib mineral oil (No. 9B060) that was used to establish the 95% confidence interval for Squibb mineral oil. Unfortunately, after two rate-zonal experiments with this batch of oil, we had depleted our supply of Squibb mineral oil and were unable to get more oil from this batch. Hoping that we might find a similar cell distribution to that which had been obtained from batch No. 9B060, we purchased the only Squibb mineral oil available from our supplier (batch No. 0B042). Use of this batch of mineral oil resulted in a peritonitis that was comprised of 1.9% lymphocytes, 6.2% neutrophils and 91.9% macrophages. As can be seen, this cell distribution for Squibb batch No. 0B042 falls widely outside of the 95% confidence interval established for batch No. 9B060 and provides so few lymphocytes as to make lymphocyte purification much more difficult. This example simply reinforces the argument for using a chemically defined hydrocarbon in order to standardize this test system.

Isopycnic Centrifugation

After isopycnic centrifugation, the peritonitis cells from tetramethylpentadecane, squalane and Squibb mineral oil had rather similar distributions on the isopycnic density gradients (Text-fig 1). In repeated experiments with all three oils, the macrophages were resolved into two modal populations. The first of these, hereafter referred to as *light macrophages*, came to their densities in fractions 2–4 from the isopycnic gradients. The other modal population of macrophages, hereafter referred to as *dense macrophages*, were found in fractions 6–10. Microscopic examination revealed that light macrophages (Fig 1) differ from dense macrophages (Fig 2) in having vacuoles of oil that are larger and occupy a considerably larger proportion of the total cell volume. The low density of these cells probably results from their high lipid content. The purest fractions of light macrophages were comprised of 88.5–100.0% macrophages. As shown in Table 3, the peritonitis that results from the injection of tetramethylpentadecane contains a higher proportion of neutrophils than the peritonitides evoked by squalane and by the batch of Squibb mineral oil for which a 95% confidence interval was established. In repeated experiments using peritonitis cells from exudates induced with squalane and Squibb mineral oil, light macrophages were recovered in 98.5–100.0% purity after isopycnic centrifugation. Adulteration of the light macrophage zone from the gradient was seen only as the result of contamination by



TEXT-FIG 1—Isopycnic (density-dependent) sedimentation of guinea pig peritonitis cells from peritonitides elicited by the intraperitoneal injection of tetramethylpentadecane, squalane, and Squibb mineral oil batch No. 9B060. In all experiments, 11–24 × 10^6 peritonitis cells (see text) were layered over linear, 7.8 cm, 5.5–43.0% w/w gradients of Ficoll in tissue culture medium. Centrifugation was carried out at 800 *g* (measured at sample-gradient interface, 18.2 cm from center of revolution) for 90 minutes at 4.0 C. As described in text, the macrophages are found to contain two modal populations: light macrophages (fractions 1–4) and dense macrophages (fractions 6–10). Lymphocytes resemble dense macrophages in density and are consequently not separated from dense macrophages by isopycnic centrifugation. Neutrophil zone broadly overlaps dense macrophage and lymphocyte zones, extends into the adjacent, denser fractions from the gradient and frequently has a long, trailing tail extending up into light macrophage zone.

neutrophils and was therefore seen in isopycnic separations only when those oils (tetramethylpentadecane, Drakeol 6VR, Marcol 52, hexadecane, etc) were used that were observed to elicit larger neutrophilic responses.

The modal populations of dense macrophages, lymphocytes and neutrophils overlapped broadly (Text-fig 1). After isopycnic centrifugation, the dense macrophages were found in a broad zone with the modal population being found in fraction 8 (±1 fraction); this

corresponds to a density of 1.102 g/ml. Lymphocytes had an identical modal density and an almost identical distribution. No significant difference was found in comparing the lymphocytes elicited by injecting different oils. After isopycnic centrifugation, neutrophils did not exhibit a well-defined modal density but were distributed in a broad zone of the gradient including both the dense macrophages and the adjacent, denser fractions from the isopycnic gradient. When neutrophils were present in large numbers, as in the hexadecane-induced peritonitis, a long, low tail of the neutrophil peak not uncommonly extended up into the less dense fractions of the gradient. This unfortunate characteristic of neutrophils is interpreted as constituting another reason for the choice of squalane, instead of tetramethylpentadecane or hexadecane, for routine work.

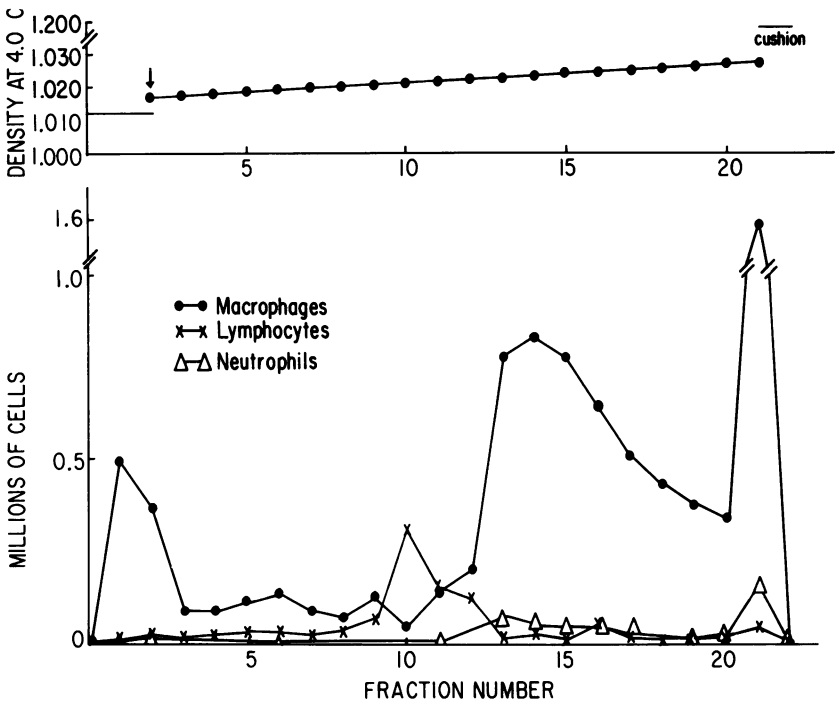
Rate-Zonal Centrifugation

The modal population of lymphocytes exhibited an effective diameter of between 5.5 and 6.5 μ; the modal population of dense macrophages, between 7.0 and 8.0 μ. Lymphocytes always traveled as a well-defined peak; more than 80% of the recovered lymphocytes (Fig 3) were located in two or three adjacent 4-ml fractions from the 80.5-ml rate-zonal gradients. Dense macrophages exhibited a sharp modal peak with a characteristic, gradually descending leading edge comprised of larger macrophages. It was not possible to calculate a valid effective diameter for the light macrophages since those entering the gradient were very heterogeneous with respect to density. A considerable proportion of the light macrophages were less dense than the top of the gradient; these were collected in fraction 1 (the starting sample suspension) and fraction 2 (the sample-gradient interface). Those light macrophages that entered the gradient decelerated and came to rest as they approached their respective densities in the early fractions from the gradient.

Simulation of several rate-zonal separations showed that if centrifugation was carried out for a sufficient period of time for the modal population of lymphocytes to reach fraction 10 (Fig 3) on the described gradient, the modal population of macrophages would be be well separated from the lymphocytes and located between the lymphocyte zone and the cushion. Aggregates would be found on the cushion (fraction 21). The forces and durations of centrifugation used to effect this separation of the peritonitis cells from the different peritonitides are listed in Table 2.

The separation of the sample suspension (Fig 4) achieved by rate-

zonal centrifugation is exemplified by a rate-zonal separation of squalane-induced peritonitis cells (Text-fig 2). Microscopic examination of the macrophages in the sample zone, at the sample-gradient interface and in the early fractions from the gradient after centrifugation shows that they are the same lipid-engorged macrophages described as light macrophages in the isopycnic separation experiments (Fig 1). Similarly, the macrophages found in fraction 13 and subsequent fractions resemble those macrophages previously termed dense macrophages. Lymphocytes were concentrated in fraction 10 (±1 fraction); however, they were invariably adulterated with variable numbers of macrophages resembling light macrophages. Neutrophils, usually present in small numbers in those peritonitides resulting from the injection of squalane and Squibb mineral oil (batches No. 9B060 and 0B042), were located in the dense macrophage zone. Neutrophils



TEXT-FIG 2—Rate-zonal separation of cells from squalane-induced peritonitis. In this experiment, 12.3 × 10^6 peritonitis cells were layered over linear, 12.3 cm, 2.6–5.5% w/w gradient of Ficoll in tissue culture medium. Centrifugation was at 54.7 g (measured at sample-gradient interface, 13.7 cm from center of revolution) for 30 minutes at 4.0 C. Well-defined zones containing light macrophages (fractions 1–2), lymphocytes (fractions 10–12), dense macrophages (fractions 13–20), and aggregates (fraction 21, gradient-cushion interface) are observed.

from peritonitis cell suspensions, as well as those from disaggregated solid tumors,[9] are frequently involved in aggregates and are therefore often found in the aggregates that have sedimented to the cushion (fraction 21) in rate-zonal centrifugation experiments.

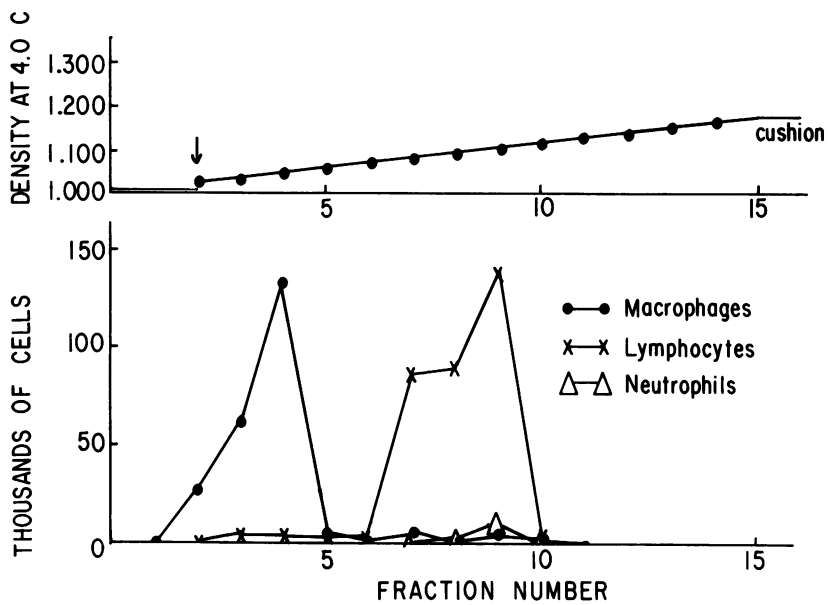
Rate-Zonal Separation Followed by Isopycnic Separation

As noted above, those macrophages sedimenting in the lymphocyte peak are engorged with oil and are of large diameter. If the density of these macrophages was similar to the modal density of lymphocytes and dense macrophages (1.102 g/ml), their larger diameters would have caused them to sediment more rapidly than the lymphocytes; therefore, it could be hypothesized that these large macrophages that were found among the lymphocytes must be less dense than the lymphocytes, and it should be possible to separate them from the lymphocytes by isopycnic (density-dependent) centrifugation.

Accordingly, fraction 10 from a rate-zonal separation of squalane-induced peritonitis cells was layered over an isopycnic gradient and centrifuged isopycnically (800 *g* for 90 minutes). The isopycnic gradient was collected, cell counts done and differential counts were performed as described above. The separation achieved by this two-step procedure is shown in Text-fig 3. In the two-step separation experiments (Text-fig 3), macrophages were recovered in 97.0% purity; lymphocytes, in 99–100% purity. Repeated experiments with squalane-induced peritonitis cells gave similar results. Two-step purifications of the peritonitis cells elicited by other oils were not attempted but should be equally as feasible.

Recovery of Cells

After isopycnic centrifugation, 51.3–83.9% of the cells layered over the gradient in the starting sample suspension were recovered in the fractions from the gradients. There appeared to be a preferential loss of macrophages. Attrition of cells appeared to be quantitatively independent of the oil used to elicit the peritonitis. If all of the experiments on isopycnic cell separation are taken together, the cell recovery was 41.7–80.4% of the macrophages, 95.1–98.0% of the lymphocytes and 60.3–91.6% of the neutrophils in the starting sample suspension. Fractions 1 and 2 from the isopycnic gradients often contained small numbers of lysed cells that appeared to be macrophages (usually with adherent vacuoles of oil) and were not included in the 200-cell differential count. Tiny particles interpreted as being the remains of lipid vacuoles from disrupted macrophages were also present in frac-



TEXT-FIG 3—Rate-zonal separation followed by isopycnic separation. In this experiment, fraction 10 from a rate-zonal separation of squalane-elicited peritonitis cells (Text-fig 2) was layered over isopycnic gradient and centrifuged at 800 *g* for 90 minutes as in Text-fig 1. Lymphocytes were separated from light macrophages, which are found in Fraction 10 from rate-zonal gradients. Using this two-step procedure, macrophages were recovered in 97.0% purity; lymphocytes, in 99–100% purity.

tions 1 and 2. More than 98% of the cells in fraction 3 and subsequent fractions excluded trypan blue.

After rate-zonal centrifugation, 61.4–87.6% of the cells layered over the gradient in the starting cell suspension were recovered in the fractions from the gradient. After rate-zonal centrifugation, attrition of cells again was found not to be correlated significantly with the oil used to induce the peritonitis. Cell recovery calculated as before was macrophages, 42.5–90.2%; lymphocytes, 97.3–99.6% and neutrophils, 68.0–91.5%.

Possible Causes of Cell Loss. The wall effect artifact is always present in gradient centrifugation using cylindric centrifuge tubes and undoubtedly accounts for some cell loss. In earlier work with other cell suspensions, we have routinely recovered 80–95% of the cells in the starting sample suspension. After staining with crystal violet, cells have been seen, with an inverted microscope, to be adhering to the walls of the centrifuge tube, and it has been assumed that most cell loss could be attributed to wall effect. The wall effect artifacts have been reviewed by others.[15,22] Loss of macrophages in the experiments re-

ported here was greater than usual and is probably the result of an unusual degree of fragility.

Error in Sampling and in Cell Identification

In order to evaluate experimental error in cell identification, differential cell counts were made independently by two of the authors on 500 cells from the same slide. Differences between investigators were found to be not significant ($P < 0.05$, χ^2 test). Typical results are given in Table 4. To examine another component of sampling error, differential counts were made of 500 cells from each of two microscopic slides prepared from the peritonitis cell suspension of the same guinea pig. Typical results are given in Table 5. A χ^2 analysis demonstrated that this factor was also not significant.

Discussion

The experiments reported here were designed to purify lymphocytes and macrophages from oil-induced peritonitis in guinea pigs using gradient sedimentation. Zbar *et al* [13] recently separated cells from oil-induced peritonitis in guinea pigs using isopycnic centrifugation in discontinuous albumen gradients; they obtained macrophages in 87% purity and lymphocytes in 45% purity. The methods reported in this paper using programmed gradient sedimentation permit both lymphocytes and macrophages to be recovered in consistently greater than 98.5% purity.

Many other approaches to the purification of cells that are active in immune processes have been developed. Macrophages commonly

Table 4. Differences Between Counts by Two Observers*

Chemical	Type of cell (%)			χ^2 value
	Macrophages	Lymphocytes	Neutrophils	
Squalane				
Observer 1	75.0	11.2	13.8	
Observer 2	74.0	12.7	13.3	0.5444
Hexadecane				
Observer 1	19.6	6.5	73.9	
Observer 2	21.3	5.6	73.1	2.5228
Drakeol 6VR				
Observer 1	68.4	7.2	24.4	
Observer 2	65.6	5.8	28.6	2.7105

* Five hundred cells were counted by each of two observers. Before calculating percentages, the lysed cells and red blood cells were substracted from the total. Lysed cells and red blood cells generally constituted 5–10% of the total number of cells counted.

Table 5. Differences in Slide Preparation*

Chemical	Type of cell (%)			χ^2 value
	Macrophages	Lymphocytes	Neutrophils	
Squalane				
Slide 1	78.2	10.2	11.6	
Slide 2	75.0	11.2	13.8	1.5040
Hexadecane				
Slide 1	17.9	5.4	76.7	
Slide 2	19.6	6.5	73.9	1.1236
Drakeol 6VR				
Slide 1	70.4	6.8	22.8	
Slide 2	68.4	7.2	24.4	.4724

* In evaluating differences resulting from slide preparation, 500 cells from each of two microscopic preparations from the same peritonitis cell suspension were counted. The lysed cells and red blood cells generally constituted 5–10% of the total number of cells counted and were subtracted from the total cell count before percentages were calculated.

have been purified by taking advantage of their proclivity to adhere to glass and plastic surfaces.[23,24] Lymphocytes have been separated from other nucleated cells by passage of the suspended starting mixture of cells through columns packed with glass wool [25] or nylon fibers.[26] Immune reactive cells have been purified from mixtures of cells by passage through columns of beads coated with specific antigens.[27,28] Each of these methods of purification takes advantage of a particular characteristic of the desired cell type—*ie,* adherence to glass or plastic, immune status or affinity for nylon fibers, and is uniquely suited for certain specific applications. Comparison of these technics is difficult since each selects a different subpopulation of the desired cell type, each is specifically suited for certain purposes, different criteria (such as morphology, plaque formation, colony formation) are used to assess the extent of purification, and many of the reports have not included data regarding the proportion of the starting sample suspension that is recovered in the purified fractions.

Bosman and Feldman [29] have recently analyzed the cellular composition, morphology and the tissue sources of cells active in some models of cellular immunity. The potential significance of a method for obtaining purified macrophages and lymphocytes from peritonitis cells has been pointed out by several investigators. Zbar *et al* mixed the separated upper, middle and lower zones from the gradient with transplantable tumor cells before innoculating the mixtures into isogenic recipient guinea pigs; cells in the lower zone, containing 45% lymphocytes, were more potent than the cells in the upper zone, containing

only 9% lymphocytes, in suppressing tumor transplantation. Similar differences in the potency of delayed skin reactions to tumor were observed when cells from the different density zones of the gradient were used. Nomoto, Gershon and Waksman [30] have studied cellular immunity to tumors in hamsters, using peritonitis cells. They point out that "the relative importance of the specific lymphocytes and activated macrophages in the rejection process, however, remains to be defined." [30] Again, this question could be approached more specifically with peritonitis cells purified as described in this report.

The Hellstroms and their colleagues have demonstrated cellular immunity to autochthonous tumors both in humans and in numerous models using experimental animals.[31–33] They have emphasized that "increased understanding of host immunity against antigenic tumors is fundamental to any approach to tumor prophylaxis or therapy by immunological means." [32] Fass *et al* [34] have shown that sustained clinical remissions after therapy for Burkitt lymphoma correlates with development of positive delayed hypersensitivity to intradermal injection of lymphoma extracts.

The technic described in this report should provide an additional tool for those investigators who are studying the roles of specific cells in the mechanisms of cellular immunity.

Summary

Isopycnic centrifugation and programmed rate-zonal centrifugation were used separately and in sequence for the purification of lymphocytes and macrophages from oil-induced peritonitis in guinea pigs. Using the chemically pure hydrocarbon squalane to induce peritonitis, both lymphocytes and macrophages could be obtained consistently in 98.5–100% purity. An analysis of the cellular composition of the peritoneal exudate induced by Squibb mineral oil batch No. 0B042 showed that the distribution of cells fell outside of the 95% confidence interval established from previous work using a different batch of Squibb mineral oil. These results are consistent with Wilner's report of different biologic activities from different batches of the same mineral oil and lead to the suggestion that a chemically pure hydrocarbon should be used consistently in order to provide a constant experimental system. Of the examined hydrocarbons, squalane induced a peritonitis that was most readily purified and that contained only a small proportion of neutrophils. The potential significance of this separation procedure is discussed and contrasted with the other available means of obtaining purified lymphocytes and macrophages.

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[*Illustrations follow*]

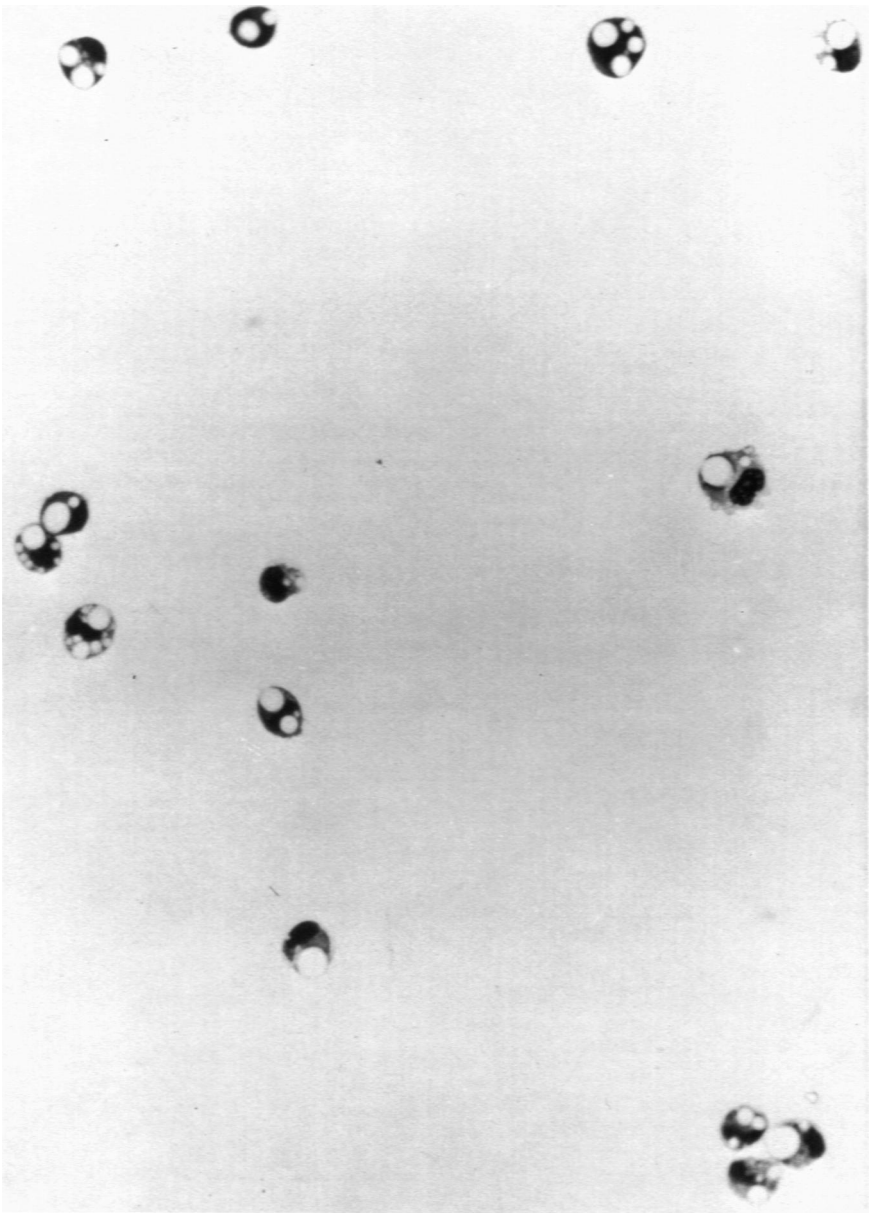

Fig 1—Light macrophages from fraction 2 after isopycnic separation of squalane-induced peritonitis. Light macrophages are typically large in diameter and distended with vacuoles of oil (Wright-Giesma stain, × 250).

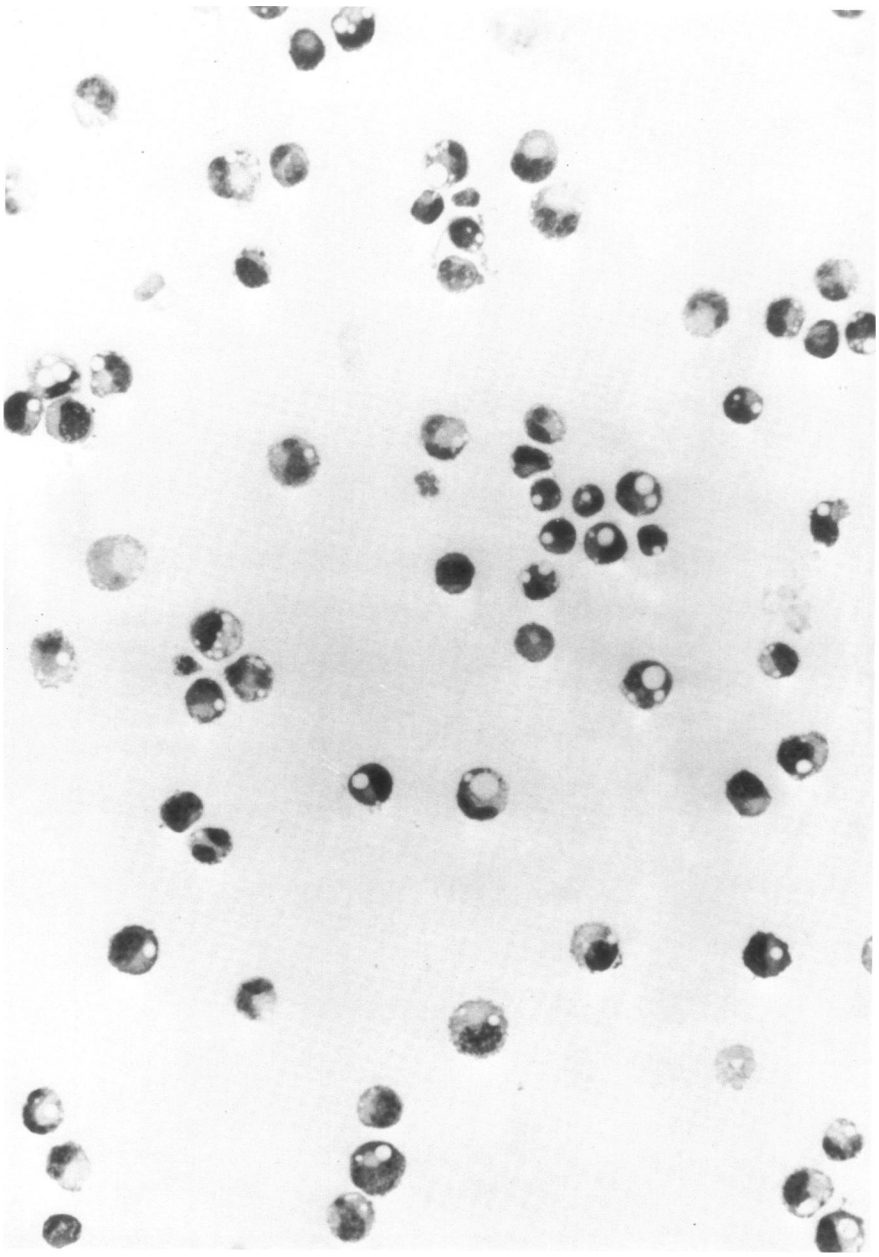

Fig 2—Dense macrophages from fraction 15 after rate-zonal separation of squalane-induced peritonitis. Dense macrophages from this zone of rate-zonal gradient have been separated from lymphocytes primarily because of difference in diameter between the two cells. It can be seen that macrophages from this zone of gradient generally contain smaller vacuoles of oil than light macrophages and that oil occupies a smaller fraction of total cell volume (Wright-Giemsa stain, × 250).

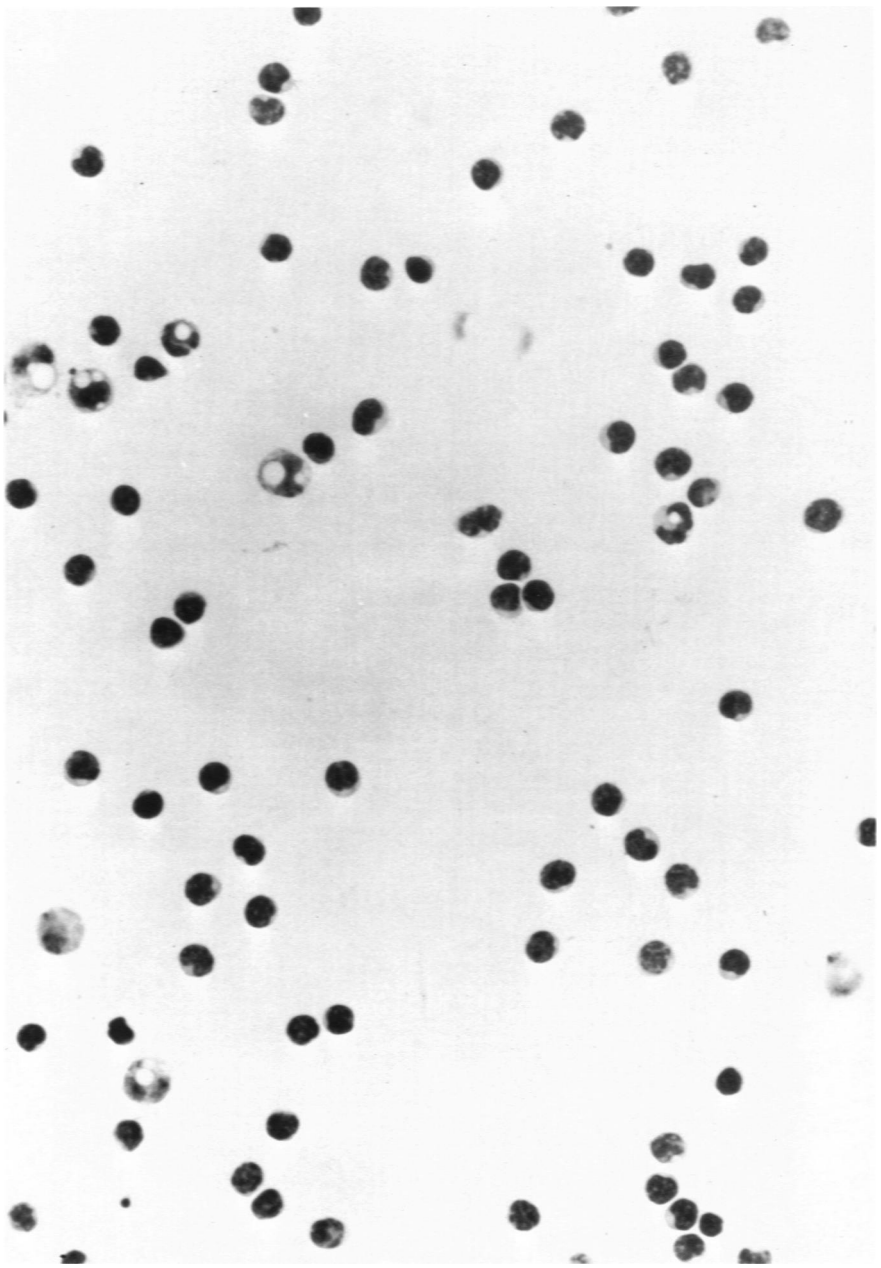

Fig 3—Fraction 10 from rate-zonal separation of squalane-induced peritonitis. This fraction contains modal diameter of lymphocytes as well as some macrophages (Wright-Giemsa stain, × 250).

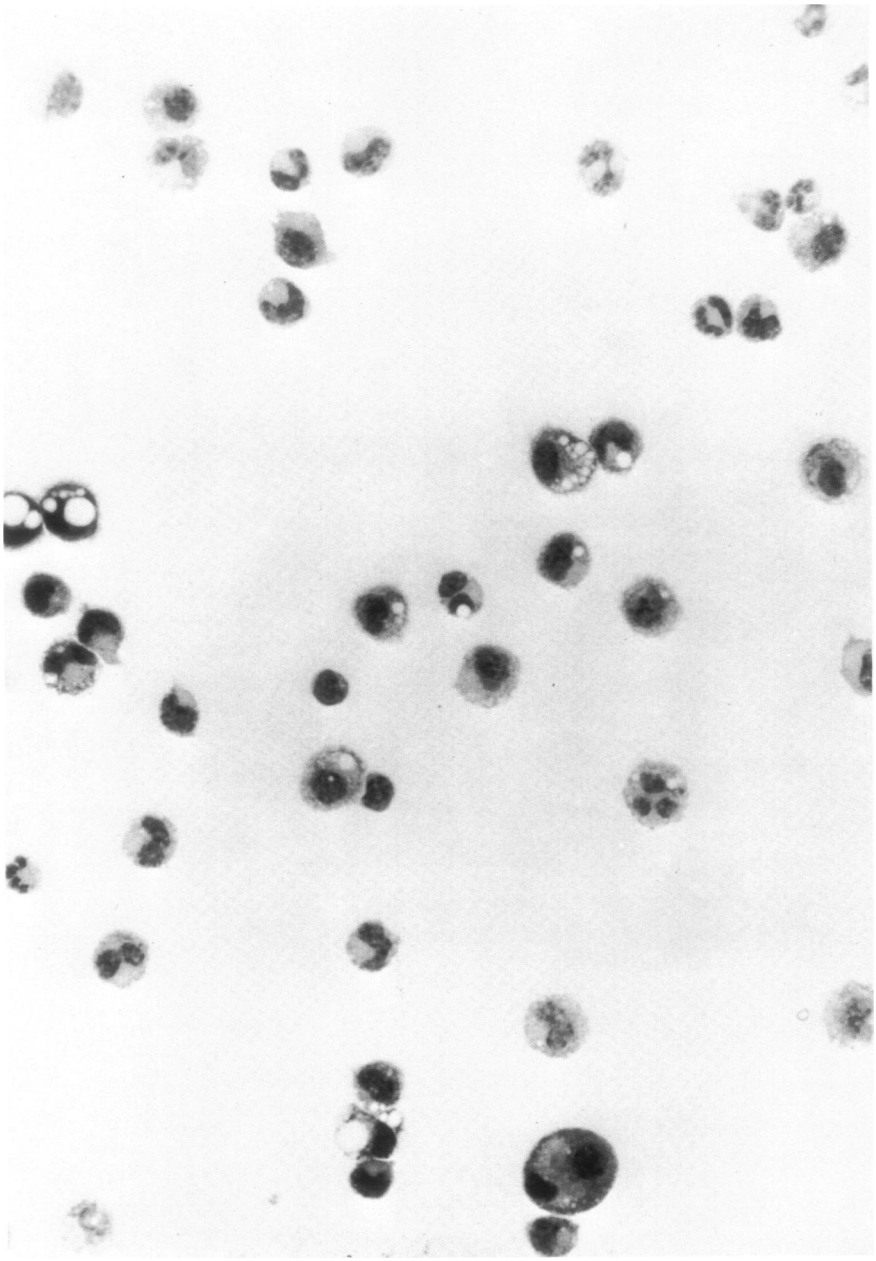

Fig 4—Starting sample suspension from squalane-induced peritonitis (Wright-Giemsa stain, × 250).